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FINAL REPORT

Report Prepared by C. H. Wilhelmj, M. D. and A. B. Vialpando

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Contractor: The Creighton University, Omaha, Nebraska

Principal Investigator: H. C. Struck, Ph.D.

Assistants: Arthur B. Vialpando, M.S., Research Associate.
Robert A. Mitchell, M.S., Research Fellow.
Frank J. Leary, B. S., Research Fellow.
John F. Collins, B.S., Research Fellow.
Frank Tracy, D.D.S., M.D., Research Associate.
Robert Dunlay, B.S., Research Fellow.

Title of Project: The Effects of Methylene Blue on Mammalian Erythrocytes

Objectives:

1. Long term study of effects of methylene blue administration to normal laboratory animals.
2. In vitro study of the effect of methylene blue on the metabolism of the mammalian erythrocyte.
3. The production of polycythemia in dogs by repeated transfusions, and the effect of methylene blue administration to these animals.
4. Administration of methylene blue to human subjects with polycythemia vera in an effort to control the disease.

Results:

I. The effect of methylene blue in normal laboratory animals --

Originally six dogs were carefully standardized, and then given daily doses of methylene blue orally in amounts sufficient to produce marked anemia. After establishing the fact that the degree of anemia was directly proportional to the doses of dye, we continued administration without interruption, varying the dose, from time to time to demonstrate that recovery was possible and that increasing the dose would bring about increased severity of the anemia.

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During the period of severe anemia the following hematologic data were collected:

The hemoglobin content of the blood dropped from the normal of 14-16 gm.% to 3-6 gm.%, and paralleled very closely the drop in red count.

The mean cellular hemoglobin remained in the normal range.

The mean cell volume rose to 90-105 cubic micra from the normal of 70-75.

The leucocyte count remained in the normal range throughout.

There were numerous normoblasts in the peripheral blood, ranging up to 60% of the total nucleated cells.

The platelet count remained within the normal range.

When the erythrocyte count dropped below 1,000,000, the reticulocyte count rose rapidly to between 15-25%.

The fragility of the cells increased, hemolysis beginning at 0.6-0.7% saline, and being complete at 0.4-0.44%.

The bone marrow became hyperplastic with normoblastic proliferation. The H:E ratio dropped from normal values of 3.6:2.2:1 to 1.1-0.4:1.

Excretion of fecal urobilinogen increased as much as 600%, but decreased to near normal values as the erythrocyte level of the blood stabilized at any level.

This evidence shows that the anemia is hemolytic in nature, and we were unable to detect any signs of bone marrow depression during the 42 months in which these dogs were maintained in a nearly constant state of anemia.

The animals have been sacrificed and microscopic study of their tissues was made. The liver, kidney, and especially the spleen show moderate deposition of hemosiderin pigment but no further pathology attributable to the dye was found.

We also gave massive repeated doses of the dye to dogs and rats, and were able to produce fatal anemias in these animals by these means. No pathologic changes other than the extreme anemia were found in these animals.

II. The effect of methylene blue on the metabolism of mammalian erythrocytes --

A. Erythrocyte Cholinesterase --

It has been reported that methylene blue is a potent anticholinesterase drug, and since it has also been reported that inhibition of erythrocyte cholinesterase causes hemolysis, we attempted to determine whether this action of the dye is responsible for the observed hemolysis produced by the dye.

To accomplish this purpose, we compared the action of methylene blue and di-isopropyl fluorophosphate (DFP) in dogs.

The methylene blue caused severe anemia which was of the characteristic hemolytic type. The doses used were sufficient to lower the red count to dangerous levels in a week to ten days. There was marked reticulocytosis, and the bone marrow showed marked hyperplasia. The erythrocyte fragility was greatly increased.

In contrast with these results, the DFP caused only a relatively mild anemia. In no case were we able to reduce the blood count below 4.5 million regardless of the length of time the drug was administered. The dose was the maximum tolerated dose, approximately 0.3 mg./kg. Increasing the dose beyond this in other dogs resulted in changes in the nervous system and myoneural junctions which have been described by other workers. Even such doses, however, did not cause the red count to drop below the figure mentioned above. The fragility of the erythrocytes during treatment with DFP did not change from the normal. The bone marrow showed stimulation, although the reticulocytes of the peripheral blood never rose above 2%, in contrast to the situation during administration of methylene blue when the reticulocyte count rose to as high as 25%. The erythrocyte cholinesterase content dropped to nearly zero during DFP administration. During treatment with methylene blue, the erythrocyte cholinesterase content tended to fall at first, but rose promptly when reticulocytosis occurred, and roughly paralleled the reticulocyte count.

We feel that this demonstrates quite clearly that, although the dye is without

question an anticholinesterase agent, this action is not a major factor in the production of the anemia. We are uncertain as to the reason for changes in the bone marrow which occur under the influence of DFP, since there is stimulation in the same direction as during methylene blue administration. We suggest that possibly the intense anticholinesterase action of the DFP allows constriction of the marrow sinusoids, causing a certain degree of hypoxia, thus stimulating erythropoiesis. This same constriction of the sinusoids could prevent the release of the products of erythropoiesis into the peripheral blood, thus causing the mild anemia observed when this drug is given.

B. Carbohydrate metabolism in the erythrocyte --

Others have shown that normal phosphorylative glycolysis is necessary to prevent hemolysis. Using cell swelling during a one hour period as an index of hemolysis, erythrocytes suspended in ~~Arcob-Hinger-Phosphate~~ solution with glucose substrate and methylene blue showed a 10% increase in volume over controls in which no methylene blue was present. This swelling is a function of the temperature, indicating that the phenomenon is the result of an enzymatic, active process.

In confirmation of others, we have found that the rate of oxygen uptake of washed erythrocytes (glucose substrate) is increased about ten-fold by the addition of methylene blue in concentrations of the order of $1:10^5$ to $1:10^6$. We have also found that in the presence of the dye approximately three atoms of oxygen are utilized per mol of glucose. The rate of glucose disappearance, however, is not altered by the dye. In controls, without dye, glucose was quantitatively converted to lactate plus pyruvate. In the presence of the dye, less than one mol of these substances was found per mol of glucose utilized. Further, in the presence of methylene blue, only negligible amounts of lactate could be found, pyruvate being the principle product of the oxidation. These experiments suggested three possible sites of action of the dye:

1. Methylene blue might inhibit utilization of glucose, since less triose was formed per unit of time. This cannot be true, however, since it was found that the rate of glucose disappearance was the same whether or not methylene blue was present.

2. Methylene blue might convert the oxidation of glucose from the Embden-Meyerhoff route to the Warburg-Christian-Dickens cycle. In testing this hypothesis, we found that neither cells nor hemolysates were capable of metabolizing pentoses or pentose phosphates to a significant extent.

3. The methylene blue might catalyze the transformation of trioses to four-carbon molecules. We have evidence to present which can apparently be interpreted in this way.

Since suspensions of erythrocytes in the presence of glucose and methylene blue failed to yield significant amounts of lactate as the end product, it was decided to investigate the lactate metabolism of the cells in the presence and absence of dye. Standard Warburg methods were used for determination of oxygen and carbon dioxide exchange. It was found, first, that cells washed three times with substrate-free Krebs-Ringer-Phosphate solution still retained small amounts of both glucose and lactate. When methylene blue was added to such a nearly substrate free suspension, there was no difference in oxygen uptake from the controls without dye over a period of several hours. Then there occurred a rather sudden and marked increase in QO_2 in suspensions with methylene blue. This continued for about 90 minutes, after which no further oxygen was utilized. The result is a biphasic curve of oxygen uptake when plotted.

At the end of the reaction the contents of the vessels were found to be completely hemolysed. Addition of lactate only had no effect on the QO_2 (it remained at the same level as the controls without added lactate or methylene blue). But, the addition of lactate plus methylene blue always resulted in biphasic curves of oxygen uptake, and this occurred over a wide range of lactate

concentrations. In every case the curves are similar, although the amount of oxygen utilized is increased and the interval before the "burst" of oxygen consumption is shortened by increasing the lactate concentration.

Red cell counts were done periodically on the contents of the Warburg flasks during the reaction. There was no reduction in the number of intact cells during the first phase, but beginning with the onset of the second phase, there was a rapid decrease. When oxygen uptake ceased, hemolysis was complete. There was no hemolysis in controls without the dye.

Ramsey and Warren have shown that there is sometimes a sudden increase in oxygen consumption during hemolysis under certain conditions. Since we found an increase in QO_2 occurring simultaneously with hemolysis, we performed the following experiment: Washed erythrocytes were suspended in hypotonic Krebs-Ringer-Phosphate solution, and were hemolyzed in the Warburg vessels by addition of distilled water containing lactate and methylene blue from the side arms. This brought about practically instantaneous hemolysis. We did not obtain a "burst" of oxygen uptake. Instead, we found the same biphasic curve of reaction as in control suspensions in isotonic medium. From this we conclude that cell rupture, per se, does not bring about the increased oxygen uptake seen during the second phase of the reaction.

Analyses for pyruvate and lactate showed that the increase in oxygen consumption during the first phase can be accounted for quantitatively by conversion of lactate to pyruvate. It is interesting that accumulation of a certain amount of pyruvate seems necessary for initiation of the second phase of the reaction. This amount varies somewhat from sample to sample of cells, but is approximately 5 micromols per 3 cc. of cells. The pyruvate concentration necessary is quite constant in a given sample of cells over a wide range of lactate concentrations, although accumulation occurs more rapidly at higher lactate concentrations.

When we attempted to follow carbon dioxide exchange we obtained unexpected

results. It was found that carbon dioxide was formed during the first phase of the reaction, and then seemed to disappear during the second phase. The carbon dioxide formed during the first phase could not be accounted for by oxidation of residual glucose in the cells. We have postulated that it was formed by decarboxylation of pyruvate. We have tested the ability of dog erythrocytes to decarboxylate pyruvate and have obtained CO_2 evolution from suspensions both in the presence and absence of methylene blue. The amount of CO_2 evolved was significantly increased when the dye was also present.

The disappearance of carbon dioxide during the second phase can be explained if one assumes that CO_2 is fixed to pyruvate, forming oxalacetate. This reaction would require the presence of oxalacetic carboxylase. We have studied the ability of cells to decarboxylate oxalacetate, using this as a means of determining the presence of the enzyme. Again, standard Warburg methods were used, one set of experiments being run at pH 7.4, the other at pH 4.0. Decarboxylation proceeds in both cases, although at a much faster rate at the lower pH. Further, methylene blue accelerated the reaction by about 40% over the controls without dye.

Fixation of carbon dioxide in this manner, of course, requires energy. We postulate that the dehydrogenation of lactate, the oxidation-reduction of methylene blue, and possibly the decarboxylation of pyruvate, supply the required energy.

This evidence suggests to us that cell destruction is the result of one or more of the reactions which are initiated by methylene blue and lactate. This could be at least an important factor in producing the anemia observed in vivo when the dye is administered to animals or humans, since both lactate and dye would be available in the circulating blood. Obviously the conditions in vivo are quite different from those existing in our in vitro experiments, one major difference being the presence, in vivo, of glucose in relatively large concentrations. When glucose and lactate were combined as substrate in cell suspensions, methylene blue caused a greater increase in oxygen consumption than with glucose alone, but no

biphasic reaction was found. Hemolysis was not detected by ordinary means over a period of six hours. There was, however, marked cell swelling.

This would suggest that glucose in some manner protects the erythrocyte from hemolysis as seen with lactate plus methylene blue only. This quite conceivably explains why the hemolysis seen in vivo is slow and can readily be controlled, only a relatively few cells rupturing during a short interval rather than all.

III. Production of Polycythemia in dogs --

Three dogs have been subjected to repeated injections of saline suspensions of washed dog erythrocytes. We were able to raise the red blood counts of these dogs to 9-10,000,000 by this means. Blood volume determinations showed that this was a real increase in the number of circulating erythrocytes since the plasma volumes did not change significantly in these animals.

The polycythemic animals were then given methylene blue. It was observed that the red count could be decreased rapidly from polycythemia to anemia, again depending upon the dose of dye. Here, again, it was seen that the change dealt only with the erythrocytes, plasma volume fluctuating very little, if at all.

IV. Methylene Blue Treatment of Human Polycythemia --

Four cases of human polycythemia have been made available for this part of the work, and treatment has been carried out with the help of our department of medicine. For purposes of convenience, each case will be discussed individually here:

A. Mrs. A. C. - Age 64 -

This patient came under our care with the following blood picture.

Hemoglobin	21.5 grams percent
Red blood count	10,410,000/cmm.
White blood count	7,350/cmm.
Platelet count	374,000/cmm.
Total Blood volume	126 cc./kg.

Total red cell volume 87 cc./kg.

Plasma Volume 39 cc./kg.

Bone marrow showed normal appearing erythropoiesis and myelopoiesis with an H:E ratio of 3.8:1.

The patient's symptoms at this time included marked weakness, dizzy spells, numbness and tingling of the fingers, and dull, bitemporal headaches.

When the diagnosis was definitely established, this patient was placed on chocolate coated methylene blue tablets, 1000 mg./day. Within one week, the red blood count fell to 8,500,000 and stabilized there despite continued administration of the dye. The dosage was then increased to 2000 mg./day. This resulted in a gradual decrease in the erythrocyte count to 5,000,000/cmm. within three week's time. It was then found that a daily dose of 600 mg. was sufficient to keep the erythrocyte count at this normal level.

The condition of the patient is now subjectively and objectively much better. The above-mentioned symptoms have either disappeared entirely or have become very minor. All components of the blood picture have been returned to a normal level by this treatment. A mild episode of gastric irritation which was apparently brought on by the dye has been controlled by changing to enteric coated tablets of methylene blue, generously supplied by Abbott Laboratories.

B. Mrs. L. M. - Age 71 -

This patient was a known polycythemic for one year prior to being sent to us. She had been previously treated with phlebotomies and one course of urethan. Her hematologic workup on coming to us showed:

Hemoglobin	20 grams percent.
Red blood count	16,350,000/cmm.
White blood count	26,000/cmm.
Platelet count	650,000/cmm.
Total Blood Volume	140 cc./kg.
Bone marrow showed normal erythropoiesis.	

Her symptoms included weakness, dizziness, numbness and tingling of fingers, palpitation, angina pectoris, and paroxysmal dyspnea. Her appearance was markedly dusky and cyanotic.

She was placed on 1000 mg. chocolate coated methylene blue per day. When it was seen that the erythrocyte count was not rapidly controlled by this dose, an attempt was made to increase the dose, but the patient experienced moderate gastric irritation, and we were unable to maintain an increased dose. During this time, the patient suffered an anterior myocardial infarction and it was decided to do repeated venesections in order to more rapidly control the polycythemia, then to attempt to maintain the red count at as nearly normal a level as possible with the experimental drug. Accordingly, 2500 cc. of whole blood was withdrawn within one week's time. The red blood count was reduced to 6,370,000 by this means, and the patient was continued on a dose of 900 mg. enteric coated methylene blue per day.

The patient's dusky, cyanotic appearance cleared, her strength increased, and she was able to return to her simple household duties. She has continued on the 900 mg. dose for four months, her red blood count at this writing is in the vicinity of 7,500,000. The platelet and white blood counts haven't changed significantly since the initiation of therapy. If the count rises further, we plan to use greater doses of the enteric coated material in order to avoid gastric irritation.

C. Mr. D. G. -- Age 38 --

This man was diagnosed as having polycythemia approximately one year prior to being referred to us. He had two courses of P₃₂ with only fair results prior to coming under our care. Studies of his blood revealed:

Hemoglobin	15 grams percent.
Red blood count	8,800,000/mm.
White blood count	6,600/mm.
Platelets	115,000/mm.

Total Blood Volume 78 cc./kg.

Bone marrow showed normal appearing erythropoiesis.

His symptoms included the triad of weakness, dizziness, and dull headaches.

He was placed on 900 mg. enteric coated methylene blue per day. The red blood count fell slowly to 4,500,000/cmm. over a period of six weeks. He has been maintained for six months on a dose of 600 mg./day. He is at present entirely asymptomatic.

D. Mr. D. B. - Age 40 -

This man came to us after obtaining only fair results from two courses of P₃₂ during the 18 months since diagnosed. His blood picture showed:

Hemoglobin	17.5 grams percent.
Red blood count	7,350,000/cmm.
White blood count	7,150/cmm.
Platelets	148,000/cmm.
Total Blood volume	88.5 cc./kg.

Bone marrow showed normal appearing erythropoiesis.

The triad of weakness, dizziness and headache were about his main symptoms.

He was placed on 600 mg. enteric coated methylene blue per day. His erythrocyte count was reduced to 5,000,000 during the first six weeks of therapy. He has continued for four months on the 600 mg. dose and is now entirely symptom-free.

V. Discussion --

Our studies concerning the long-range efficacy of methylene blue treatment in polycythemia vera will continue. The studies thus far suggest that the drug will be most efficacious in those polycythemias in which only the numbers of circulating erythrocytes, and none of the other formed elements of the blood, are increased.

The Navy contract has enabled us to establish the safety of the drug for

human use, to collect evidence pointing to its mechanism of action, and to apply the data obtained to the treatment of a human disease. Perhaps most important of all, the contract has made it possible for six young men to receive valuable basic training in research, and the nation's reserve of scientific manpower has been increased.

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